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(57) Abstract

A novel bacterium named *Pseudomonas cepacia* G4 5223 Phe(1) has the desirable property of constitutively degrading hazardous chemicals, for example trichloroethylene, 1,1-dichloroethylene, *cis*-1,2 dichloroethylene, *trans*-1,2-dichloroethylene, toluene, phenol, *o*-cresol, *m*-cresol, *o*-xylene, and benzene, to harmless chemical entities. This microbe, and mutants thereof which retain the constitutive degradation property of the parent, can be used in bioreactor and *in situ* processes for degrading hazardous chemical compounds.

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DESCRIPTION

MICROBIAL DEGRADATION OF TRICHLOROETHYLENE, DICHLOROETHYLENES AND AROMATIC POLLUTANTS

Background of the Invention

Groundwater contamination by organic pollutants is a subject of major concern in the United States and, increasingly, the rest of the industrialized world. The sources of pollution are almost as numerous as the types of pollutants found. Sources range from agricultural uses, injection wells, underground storage tanks and hazardous waste sites, to illegal dumping in localities with no prior history of toxic chemical exposure. In monitoring chemical waste disposal sites within the United States, pollutants have been categorized according to the frequency of detection of certain chemical classes and specific chemicals within that class. The priority pollutants are divided into volatiles, acid extractables, pesticides and base/neutrals. Volatiles are the most frequently detected group, owing largely to their widespread use as solvents. The most often encountered components of these volatile chemical pollutants in 1986 were, in order of frequency, tri-chloroethylene, tetrachloroethylene, trans-1,2-dichloroethylene, 1,1-dichloroethylene, (ranked first, second, third and fifth respectively of all volatiles detected (Rajagopal, R. 1986. Environ. Prof. 8:244-264). The most prevalent of these, trichloroethylene (TCE), has been in use since the 1940's, and the subject of environmental and human health concerns since its discovery in a contaminated aquifer in 1979 (Muntner, J.E., and S.P. Devries, 1987. Toxics Law Reporter, Jan. 14:874).

All reports of TCE transformation by anaerobic bacteria indicate a very slow process. The rapid mineralization of TCE by aerobic bacteria has been demonstrated for several microbial isolates. All require the addition of exogenous chemicals to induce production of the requisite TCE degrading enzymes. Toluene serves to induce oxygenase enzymes of Pseudomonas putida F1 (Nelson, M.J.K., S.O. Montgomery, and P.H. Pritchard. 1988. App. Environ. Microbiol. 54:604-606) and P. cepacia G4 (Nelson, M.J.K., S.O.

Montgomery, E.J. O'Neill, and P.H. Pritchard. 1986. Appl. Environ. Microbiol. 42:383-384). These enzymes are in turn responsible for TCE degradation by these bacteria. The route of toluene catabolism by P. cepacia G4 has been shown to proceed via a monooxygenation pathway that results first in an ortho-hydroxylation of toluene (catalyzed by o-toluene monooxygenase) and subsequently a second hydroxylation ortho to the first hydroxyl to form 3-methylcatechol (Shields, M.S., S.O. Montgomery, P.J. Chapman, S.M. Cuskey, and P.H. Pritchard. 1989. Appl. Environ. Microbiol. 55:1624-1629).

U.S. Patents concerning bioremediation are as follow: 4,749,491; 4,452,894; 4,664,805; 4,477,570; 4,925,802; 4,954,258.

U.S. 4,749,491 concerns a method for stimulating indigenous bacteria to degrade chlorinated hydrocarbons through the addition of oxygen and propane or methane.

U.S. 4,664,805 concerns a method for degradation of halogenated organic pollutants through the addition of non-toxic chemical analogs with or without non-indigenous microorganisms.

U.S. 4,452,894 concerns a pure culture of a Pseudomonas spp. that can utilize a variety of chlorinated aromatic chemicals as sole sources of carbon.

U.S. 4,477,570 concerns the isolation of bacterial strains, specifically Pseudomonas cepacia var. niagarous, that degrade aromatic and halogenated aromatic chemicals.

U.S. 4,954,258 concerns the additiona of alkanes or lower alkanols to methanotrophic bacteria for the degradation of TCE.

U.S. 4,925,802 concerns a method for stimulating biodegradation of halogenated aliphatic hydrocarbons. The method uses microbes and an inducer. Specifically exemplified is the non-constitutive microbe, Pseudomonas cepacia strain G4, which is the parent of the constitutive microbe strain of the subject invention.

Current technology for the treatment of TCE-contaminated soil and water has relied primarily upon pump-and-treat systems whereby TCE is distilled away from the water under vacuum, or alternatively is air-stripped and transferred onto an adsorbent such as charcoal. Recent reviews of this subject

strongly question whether this technology alone will ever be effective in the remediation of hazardous wastes like TCE, since even long term treatments have had only modest effects on pollutant concentrations (Travis, C.C., and C.B. Doty. 1990. Environ. Sci. Technol. 24:1464-1466). In either event, the result of such treatment is simply the transfer of the pollutant to an adsorbent or to the atmosphere. The capability to destroy the contaminant at the site would represent significant environmental and economic benefits. If realized, efficient bioremediation technologies would fill this need. Major limitations to the bacterial systems described in the prior art for the bioremediation of TCE is that they all degrade TCE fortuitously. That is to say their ability to alter TCE is necessarily linked to the production of an enzyme that can accept TCE as a surrogate substrate, the native substrate being that which is used to induce the enzyme's synthesis. Due to this co-metabolic relationship, TCE cannot be degraded in the environment without the addition of an exogenous inducing substrate, because TCE does not itself induce the enzymes required for its own degradation. As a result, these prior art organisms are faced with the additional limitation of degrading TCE in the presence of the required co-substrate that competes for the same active site on the induced enzyme. In addition, this also means that the organisms are not active beyond the environmental zone that can be controlled through the addition of effective concentrations of inducer. The active bacteria are effectively "tethered" to the inducing substrate. Both of these limitations have serious implications to the design of both environmental and bioreactor applications. In addition, the application of native inducing substrates such as toluene or phenol is not possible in the environment as they are themselves pollutants.

The use of the microbe of the subject invention, advantageously, does not have the problems associated with the use of prior art microbes to remediate sites contaminated with hazardous chemicals. Briefly, the microbe of the invention, P. cepacia strain G4 5223 Phe (1), is a natural bacterial isolate that can function over a wide range of environmental conditions without the need for an added chemical inducer; it has a very high likelihood of competitive maintenance among native bacteria over the course of a given

treatment; and it has evolved to utilize and grow on many organic pollutants likely to be found at waste treatment sites.

Brief Summary of the Invention

The subject invention concerns the use of a novel bacterial strain to degrade TCE over a wide range of environmental parameters without the requirement for added chemical inducers.

More specifically, the subject invention concerns the use of the novel strain designated Pseudomonas cepacia strain G4 5223 Phe (1) to degrade hazardous chemicals, for example, trichloroethylene (TCE), cis-1,2-dichloroethylene, trans-1,2-dichloroethylene, 1,1-dichloroethylene, and aromatic chemicals, for example benzene, phenol, toluene, o-xylene, m-xylene, o-cresol, and m-cresol. The novel bacterium is fully capable of the complete removal of the aforementioned pollutants under a variety of conditions, without the need to artificially or externally influence existing conditions (i.e. induce the requisite enzymes). Without the need for exogenous chemical inducers, this novel bacterium is an attractive biodegradative agent for remediation of TCE pollution for two reasons. First, and most importantly, the organism is free of the requirement of inducer and capable of functioning under a diverse set of conditions. Second, this constitutive degrader does not require an inducer that is a co-substrate for the same enzyme required to degrade TCE; in effect, it is not subject to competitive inhibition. As such, it can be used in both in situ environmental and bioreactor remediation processes.

The subject invention also includes the genes(s) harbored by the novel bacterium which gene(s) encode enzyme(s) capable of degrading the hazardous chemicals. These gene(s) can be isolated from the parent bacterium by using well-known cloning techniques. The isolated gene(s) can be used to transform other microbes by use of well-known transfer and expression vectors. The transformed host can be used in the same manner as the parent microbe to degrade hazardous chemical compounds.

Further, the enzyme(s) responsible for the degradation of the hazardous chemical compounds can be recovered from the extracts of cultures of the novel

bacterium and used to degrade hazardous chemical compounds by procedures well known in the art.

Brief Description of the Drawings

Figure 1 Illustrates the first two oxidations performed on toluene by P. cepacia G4.

Figure 2 A presentation of the probable route of oxidation of the m-cresol analog: 3-trifluoromethylphenol (TFMP) to the yellow ring-cleavage product.

Figure 3 A portrayal of the known catabolic transformations associated with the initial enzymes of toluene catabolism in P. cepacia G4.

Figure 4 The effects of several environmental variables on the initial (i.e., over the first 1-2 hr) rate of TCE degradation by P. cepacia G4 5223 are shown.

Detailed Disclosure of the Invention

Upon contact with a culture of Pseudomonas cepacia strain G4 5223 Phe(1), or a mutant thereof which retains substantially the degradative capability of the parent bacterium, with a hazardous chemical, as defined herein, the hazardous chemical is degraded to a non-hazardous entity.

A subculture of P. cepacia strain G4 5223 Phe(1) has been deposited in the permanent collection of the Agricultural Research Service Culture Collection, Peoria, Illinois. The accession number is as follows:

Pseudomonas cepacia strain G4 5223 Phe(1)	NRRL B-18811

The taxonomy of Pseudomonas cepacia strain G4 5223 Phe(1) is as follows:

Pseudomonas cepacia G4 5223 Phe(1) is an oxidase-positive and catalase-positive gram-negative obligately aerobic rod. It reduces nitrate to nitrite, is incapable of denitrification, and is unable to ferment glucose or hydrolyze esculin. Tests for urease, gelatinase, and arginine dihydrolase activity were negative. Beta-galactosidase activity is present and substrate utilization tests showed growth on D-glucose, D-arabinose, D-mannose, mannitol, N-

acetyl-D-glucosamine, maltose, gluconate, caprate, adipate, citrate, and phenylacetate. L-malate is not utilized. Neither pyocyanin or fluorescein pigments are formed on selective media (King, E.O., M.K. Ward, and D.E. Raney. 1954. J. Lab. Clin. Med. 44:301). Pseudomonas cepacia G4 5223 Phe(1) is resistant to kanamycin sulfate (50μg/ml) in basal salts medium (Hareland, W., R.L. Crawford, P.J. Chapman, and S. Dagley. 1975. J. Bacteriol 121:272-285) containing 20mM sodium lactate as the sole carbon source and is also capable of growth with penicillin G (5mg/ml) as the sole carbon source. In addition, its demonstrated ability to store excess carbon as a microbial polyhydroxyalkanoate polyester may offer an excellent method to prolong degradative functions under the most extreme conditions.

The procedure of application of P. cepacia G4 5223 Phe(1) to the remediation of TCE-contaminated materials may be carried out through the use of various known procedures. For example the organism may be used in a bioreatcor (fixed film, fluidized bed, etc.) as well as in situ by methods generally such as those disclosed in U.S. Patent Nos. 4,749,491 and 4,588,506.

Enzyme(s) produced by the novel microbe of the invention can be recovered from the cultured cells of the microbe. The recovery process can be one in which the microbial cells at harvest are extracted and the enzyme(s) recovered by standard procedures. The resulting enzyme preparation can be used to degrade hazardous chemicals, as disclosed herein. The treatment of hazaradous chemicals with an enzyme preparation, as disclosed above, can be by use of columns and other means well known in the enzyme art. The enzyme preparation so used can be in either a crude or essentially pure form.

Novel recombinant microbes can be made by isolating the gene(s) from P. cepacia strain G4 5223 Phe(1) and transforming suitable hosts with the gene(s). The gene(s) encode enzymes which are capable of degrading hazardous chemical compounds.

A wide variety of ways are available for introducing a gene into a microorganism host under conditions which allow for stable maintenance and expression of the gene. One can provide for DNA constructs which include the transcriptional and translational regulatory signals for expression of the gene,

the gene under their regulatory control and a DNA sequence homologous with a sequence in the host organism, whereby integration will occur, and/or a replication system which is functional in the host, whereby integration or stable maintenance will occur.

Various manipulations may be employed for enhancing the expression of the messenger RNA, particularly by using an active promoter, as well as by employing sequences, which enhance the stability of the messenger RNA. The transcriptional and translational termination region will involve stop codon(s), a terminator region, and optionally, a polyadenylation signal.

In the direction of transcription, namely in the 5′ to 3′ direction of the coding or sense sequence, the construct will involve the transcriptional regulatory region, if any, and the promoter, where the regulatory region may be either 5′ or 3′ of the promoter, the ribosomal binding site, the initiation codon, the structural gene having an open reading frame in phase with the initiation codon, the stop codon(s), the polyadenylation signal sequence, if any, and the terminator region. This sequence as a double strand may be used by itself for transformation of a microorganism host, but will usually be included with a DNA sequence involving a marker, where the second DNA sequence may be joined to the expression construct during introduction of the DNA into the host.

A marker structural gene is used to provide for the selection of the host microbe which has acquired the desired nucleotide sequence (via, for example, transformation, electroporation, conjugation, or phage mediated). The marker will normally provide for selective advantage, for example, providing for biocide resistance, e.g., resistance to antibiotics or heavy metals; complementation, so as to provide prototrophy to an auxotrophic host, or the like. Preferably, complementation is employed, so that the modified host may not only be selected, but may also be competitive in the field. One or more markers may be employed in the development of the constructs, as well as for modifying the host. The organisms may be further modified by providing for a competitive advantage against other wild-type microorganisms in the field. For example, genes expressing metal chelating agents, e.g., siderophores, may be introduced

into the host along with the structural gene. In this manner, the enhanced expression of a siderophore may provide for a competitive advantage for the host, so that it may effectively compete with wild-type microorganisms.

Where no functional replication system is present, the construct will also include a sequence of at least 50 basepairs (bp), preferably at least about 100 bp, and usually not more than about 1000 bp of a sequence homologous with a sequence in the host. In this way, the probability of legitimate recombination is enhanced, so that the gene will be integrated into the host and stably maintained by the host. Desirably, the gene will be in close proximity to the gene providing for complementation as well as the gene providing for the competitive advantage. Therefore, in the event that a gene is lost, the resulting organism will be likely to also lose the complementing gene and/or the gene providing for the competitive advantage, so that it will be unable to compete in the environment with the organism retaining the intact construct.

A large number of transcriptional regulatory regions are available from a wide variety of microorganism hosts, such as bacteria, bacteriophage, cyanobacteria, algae, fungi, and the like. Various transcriptional regulatory regions include the regions associated with the trp gene, lac gene, gal gene, the lambda left and right promoters, the Tac promoter, the naturally-occurring promoters associated with the gene, where functional in the host. The termination region may be the termination region normally associated with the transcriptional termination region or a different transcriptional termination region, so long as the two regions are compatible and functional in the host.

Where stable episomal maintenance or integration is desired, a plasmid will be employed which has a replication system which is functional in the host. The replication system may be derived from the chromosome, an episomal element normally present in the host or a different host, or a replication system from a virus which is stable in the host.

The gene can be introduced between the transcriptional and translational initiation region and the transcriptional and translational termination region, so as to be under the regulatory control of the initiation region. This construct will be included in a plasmid, which will include at least

one replication system, but may include more than one, where one replication system is employed for cloning during the development of the plasmid and the second replication system is necessary for functioning in the ultimate host. In addition, one or more markers may be present, which have been described previously. Where integration is desired, the plasmid will desirably include a sequence homologous with the host genome.

The transformants can be isolated in accordance with conventional ways, usually employing a selection technique, which allows for selection of the desired organism as against unmodified organisms or transferring organisms, when present. The transformants then can be tested for degrading hazardous chemical compounds.

Suitable host cells can be Gram-negative bacteria, including Enterobacteriaceae, such as Escherichia, and other Pseudomonadaceae.

The recombinant cellular host containing the gene(s) may be grown in any convenient nutrient medium, where the DNA construct provides a selective advantage, providing for a selective medium so that substantially all or all of the cells retain the gene. These cells may then be harvested in accordance with conventional ways.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1: Aromatic degradative pathway of P. cepacia G4

The toluene catabolic pathway of P. cepacia G4 was established using mutants induced with nitrosoguanidine and heavy oxygen isotope incorporation into the catabolic products (Shields, M.S., S.O. Montgomery, P.J. Chapman, S.M. Cuskey, and P.H. Pritchard. 1989. Appl. Environ. Microbiol. 55:1624-1629). See Figure 1.

Mutants of this pathway were detectable in the presence of the fluorinated analog of m-cresol; m-trifluoromethylphenol (TFMP) (Aldrich

Chemical Co., Milwaukee, WI). Wild type cells were capable of the oxidation of TFMP to a bright yellow transformation product presumed to be 7,7,7-trifluoro-2-hydroxy-6-oxo-2,4-heptadienoic acid (Engesser, K.H., R.B. Cain, and H.J. Knackmuss. 1988. Arch. Microbiol. 149:188-197). Due to anticipated structural similarities between TFMP oxidation products and intermediates of the toluene pathway, it was predicted that mutants failing to produce yellow color with TFMP would lack either the cresol monooxidation or catechol dioxygenation functions. See Figure 2.

Mutants were characterized according to their ability to grow with different aromatic substances and their reaction with TFMP as shown in Table 1.

Table 1. Mutant Phenotypes

Strain	Genotype	TFMP Color Reaction[a] Induced with:		Growth with:			
		Tol[b]	Phe	Tol	Phe	o-Cre	m-Cre
G4	wild type	+	+	+	+	+	+
G4(100)	tomA	-	-	-	-	-	-
G4(100R1)	tomA Revertant	+	+	+	+	+	+
G4(102)	tomB	Br[c]	Br[c]	-		+[d]	-
G4(103)	tomC	Ye[c]	+	-	+	-	-

[a]Conversion of 3-trifluoromethylphenol (TFMP) to its yellow ring cleavage product is indicated by "+".
[b]Toluene (Tol), phenol(Phe), o-cresol (o-Cre) and m-cresol (m-Cre).
[c]TFMP conversion was not detectable in colonies due to accumulation of either brown (Br) or yellow (Ye) colored products formed in the presence of inducer.
[d]Very slow growth (5-7 days for recognizable colonies).

These classes of mutants were further characterized and confirmed by examination of the effects of mutation on whole cell oxygen uptake in the presence of different substrates and by measurement f the specific activities of enzymes in cell free extracts as shown in Table 2.

Table 2. Cell Free Enzyme Activities

Enzyme activities (nmol*min^{-1}mg protein^{-1})[a]					
	C230		Hydrolase		Dehydro-genase
Substrate	Cat	3mCat	Hms	Hod	Hms
Strain					
G4	6550	3780	112	1860	52
G4(100)	5290	1970	195	683	122
G4(100R1)	3390	5900	281	871	86
G4(102)	0.3	0.6	148	563	74
G4(103)	1680	1210	0.2	0.5	34

[a]Enzyme activities are reported as the average of duplicate determinations of cell- free extracts obtained from cells induced with toluene.
[b]Abbreviations: C230, catechol-2,3-dioxygenase, Cat, catechol; 3mCat, 3-methylcatechol; Hms, hydroxymuconic semialdehyde; Hod, 2-hydroxy-6-ketohepta- 2,4-dienoate.

Enzyme activities were derived from lactate grown cells following 6 hours exposure to air-entrained toluene vapor. Cell free extracts were analyzed for catechol-2,3-dioxygenase (Gibson, D.T. 1971. pg. 462-478. In D.J.R. Norris and D.W. Ribbons. Methods in Microbiology, 1971 Academic Press, New York), hydroxymuconic semialdehyde (Hms) hydrolase and Hms dehydrogenase activities (Bayly, R.C., and G.J. Wigmore. 1973. J. Bacteriol. 113:1112-1120).

This allowed the summation of data into the form of this comprehensive aromatic pathway cluster of P. cepacia G4 as shown in Figure 3.

Example 2: Toluene o-Monooxygenase of P. cepacia G4 is Required for TCE Degradation

Cells representative of the different mutant classes were induced using toluene and analyzed for their ability to degrade TCE. Washed cells were resuspended in basal salts medium containing 20µM TCE to a cell density that yielded an absorbance at 600 nm of 0.2. These samples were sealed in 10ml glass vials with Teflon-lined rubber septa and crimp rings. These vials were incubated inverted at 24 degrees C overnight with rotary shaking (180 rpm). TCE was extracted by injection of two ml pentane (HPLC grade, Aldrich Chemical Co.) with rotary shaking in an upright position at 180 rpm, 24 degrees C for 30 minutes. The pentane phase was transferred to GC vials with Teflon liners and analyzed on an HP 5790 gas chromatograph equipped with an autosampler and electron capture detector. Separation was achieved with a Vocol capillary column, 30 m, 0.53 mm ID, fused silica, 3.0 µm film thickness (Supelco, Inc., Bellefonte, PA #2-5320) at 50 degrees C.

Only the mutant impaired in its toluene monooxygenase activity, (tomA), failed to degrade TCE. The spontaneous revertant of this mutant: strain G4 100R1 fully regained both its ability to express the toluene o-monooxygenase as well as degrade TCE.

Table 3: TCE degradation by cells induced with Phenol or Toluene

Strain	TCE remaining (μM)[a] Induced with:	
	Phe[b]	Tol
G4	<0.02	<0.02
G4(100)	3.67 ±0.03	3.56 ±0.46
G4(100R1)	<0.02	<0.02
G4(102)	<0.02	<0.02
Uninoculated	3.72 ±0.07	

[a]Means of triplicate TCE determinations (± standard deviation) after 24 h, using either toluene- (Tol) or phenol- (Phe) induced cultures.
[b]Phenol or toluene induction of G4 was accomplished by exposing an overnight 20 mM lactate culture to 2 mM phenol or toluene. Two hr later the cells were harvested by centrifugation, washed and exposed to TCE in sealed vials with air headspaces.

Example 3: Isolation of a Tn5 Induced Toluene Monooxygenase Mutant of P. Cepacia G4

A method was developed for the isolation of transposon induced tomA mutants: Transposon mutagenesis was carried by a triparental mating: E. coli JM109 (pRZ102) (Jorgensen, R.A., S.J. Rothstein, and W.S. Reznikoff. 1979. Molec. Genet. 177:65-72) X E. coli JM109 (pRK2013) (Figurski, D.H., and D.R. Helinski. 1979. Proc. Natl. Acad. Sci. USA. 76:1648-1652) X P. cepacia G4. Selection for P. cepacia G4 containing a transposed copy of Tn5 was accomplished by growth of the mating mixture on basal salts medium containing 20 mM sodium lactate and 50 μg/ml kanamycin sulfate. Exposure to toluene vapor induced the requisite enzymes for toluene catabolism.

Colonies were transferred from the surface of the plate to nitrocellulose filters (0.45 μm pore size, Schliecher and Schuell, Keene, NH) previously soaked in an aqueous solution of 5mM TFMP and air dried. Mutants tomA and tomB (i.e. those lacking the toluene monooxygenase and catechol dioxygenase respectively) are easily differentiated as colorless colonies among a background of yellow wild type colonies. The two mutant types are readily distinguishable from one another as the catechol-2,3-dioxygenase mutant (tomB) turns brown in (>12Hr) basal salts media containing 20 mM sodium lactate and 2mM phenol (due to the accumulation and subsequent auto-oxidation of catechol). Mutants lacking toluene monoxygenase (tomA) do not.

Incorporation of TFMP in solid media was not sufficient to determine a single colony response as the color development required several minutes over which time the yellow product diffused throughout the plate. The nitrocellulose membrane lift technique was developed in response to this limitation. Colonies transferred to its surface immediately begin desiccation. Any water soluble yellow product produced by that colony does not diffuse away. The result is a distinct yellow coloration of all colonies capable of transforming TFMP to the yellow product.

Using this technique the transposon mutant, P. cepacia G4 5223 was isolated. This mutant class (typified by G4 100) lacked the ability to transform TFMP and to degrade TCE.

Example 4: Procedure for Isolation of a Constitutive TCE Degrader

Transposon (Tn5) mutants of tomA were inoculated by patching to basal salts plates containing 2 mM phenol as the sole carbon source. Rare colonies arising were picked to basal salts medium containing 20mM sodium lactate as the sole carbon source. These colonies were in turn pulled to nitrocellulose discs impregnated with TFMP (Example 3) and compared to wild type P. cepacia G4 similarly grown on this non-inducing medium. Colonies that became yellow were picked as potentially constitutive for tomA and tomB gene products. In this way P. cepacia strain G4 5223 Phe(1) was isolated.

Table 4: TCE degradation without inducer

Strain	TCE Remaining (μM[a])
No cells added	83.3 ±7.6
G4 5223	86.2 ±3.1
G4 5223 Phe(1)	1.4 ±1.4

[a]Mean TCE concentration of triplicate samples ± standard deviation after overnight incubation with the indicated strains (at a cell density equivalent to an A_{600} = 0.2).

Table 5: Enzyme induction vs. constitutivity

Enzyme Activity nmoles min^{-1} mg $protein^{-1}$					
Strain	Substrate Inducer	C230		Hms Hydrolase	
		Cat	3mCat	Hms	Hod
G4 5223 Phe(1)	none	156	50.4	0.44	2.5
G4 5223 Phe(1)	phenol	48	34.9	0.20	5.5
G4 5223	none	0.07	2.2	0	5.5
G4 5223	phenol	13.1	31.4	0	1.3
G4	none	2.1	3.7	0	3.0
G4	phenol	53.6	62.5	0.87	1.2

Abbreviations: C230, catechol-2,3-dioxygenase; Hms, hydroxymuconic semialdehyde; Cat, catechol; 3mCat, 3-methylcatechol; Hod, 2-hydroxy-6-oxohepta-2,4-dienoate.

The genetic stability of the invention strain was assessed by growing the cells under non-selective (i.e. basal salts medium with sodium lactate at 20 mM as the sole carbon source) and under selective conditions (i.e. the same

medium containing in addition 50μg kanamycin sulphate/ml) through serially diluted batch cultures, allowing 10 generations per transfer. Following the accumulation of ca. 100 generations, the resulting population of cells was tested for kanamycin resistance (in the non-selective group) and the ability to constitutively transform TFMP to a yellow product (both groups):

Table 6: Genetic Stability

100 Generation Growth on:	CFU X 10^8 on Plates Containing:	
	Lactate	Lactate + Kanamycin
Lactate	5.9	7.3
Lactate + Kanamycin	7.5	8.4

Lactate-grown colonies were picked to lactate plates containing 50μg/ml kanamycin. Those from 100 generations without selection exhibited 100% kanamycin resistance (150/150) as did those taken from kanamycin growth selection (75/75).

All colonies maintained tomA constitutivity under either growth condition (as demonstrated by pulling colonies to TFMP-impregnated nitrocellulose).

Example 5: TCE Degradation Under Ranges of Physical Conditions

In order to assess the capacity for P. cepacia G4 5223 Phe(1) to degrade TCE under anticipated ranges of environmental conditions the effects of oxygen concentration, pH, temperature and salinity were determined using a no air headspace TCE degradation assay (Folsom, B.R., P.J. Chapman, and P.H. Pritchard. 1990. Appl. Environ. Microbiol. 56:1279-1285) that mimicked a contaminated aquifer in that no gas headspaces were present. Unless otherwise noted in the salinity effect determinations, the liquid medium was the

basal salts medium buffered with Tris-HCl rather than phosphate. The results are shown in Figures 4a, 4b, 4c and 4d.

Example 6: Contitutive Degradation of 1,1-Dichloroethylene, cis-1,2-Dichloroethylene, and trans-1,2-Dichloroethylene

The range of chlorinated aliphatic substrates acted upon by P. cepacia G4 5223 Phe(1) was determined by the ability of cells to degrade 10μM TCE, 1,1-Dichloroethylene, cis-1,2-Dichloroethylene, trans-1,2-Dichloroethylene and tetrachloroethylene in 2 ml of basal salts medium without additional carbon sources in 10 ml Teflon lined vessels during an overnight incubation at 24 degrees C. Triplicate samples were analyzed by gas chromatography for appearance of detectable products as well as disappearance of parent compounds:

Table 7: Chloroaliphatic substrates of P. cepacia G4 5223 Phe(1)

	% Chloroaliphatics Remaining[a]				
	1,1-DCE	cis-1,2-DCE	trans-1,2-DCE	TCE	PCE
Uninoculated	100	100	100	100	100
G4 Uninduced	104	69	107	133	103
Phe(1) Uninduced	50	12.3[M]	ND[M]	2.0	104

[a]Percent substrate remaining as compared to uninoculated controls, abbreviations: 1,1-DCE, 1,1-Dichloroethylene; cis-1,2-DCE, cis-1,2-Dichloroethylene; trans-1,2-DCE, trans-1,2-Dichloroethylene; TCE, Trichloroethylene; PCE, Perchloroethylene; ND, Not Detectable. Starting concentration was 10 μM for all substrates.

[M]indicates that a metabolite was detected using gas chromatography.

Claims

1. A process for degrading hazardous chemicals selected from the group consisting of chloroaliphatic and aromatic chemicals which comprises contacting said hazardous chemicals with an effective degrading amount of the bacterium *Pseudomonas cepacia* G4 5223 Phe(1), or mutants thereof which retain the parental property of being constitutive for the degradation of said hazardous chemicals.

2. The process, according to claim 1, wherein said hazardous chemicals are selected from the group consisting of trichloroethylene, 1,1-dichloroethylene, *cis*-1,2 dichloroethylene, *trans*-1,2-dichloroethylene, vinyl chloride, toluene, phenol, *o*-cresol, *m*-cresol, *o*-xylene, and benzene.

3. The process, according to claim 1, wherein said hazardous chemicals are degraded *in situ* or in a bioreactor.

4. The process, according to claim 3, wherein said *in situ* degradation is selected from the group consisting of contaminated aquifers, landfills, industrial sites contaminated with hazardous chemicals, and hazardous chemicals waste collection areas.

5. *Pseudomonas cepacia* G4 5223 Phe(1), a microbe which has the property of being constitutive for the degradation of hazardous chemicals selected from the group consisting of chloroaliphatic and aromatic chemicals.

6. Enzyme(s) obtainable from extracts of the microbe defined in claim 5, wherein said enzyme(s) has the property of degrading hazardous chemicals selected from the group consisting of chloroaliphatic and aromatic chemicals.

7. Gene(s) obtainable from the microbe defined in claim 5, wherein said gene(s) encode enzyme(s) having the property of degrading hazardous

chemicals selected from the group consisting of chloroaliphatic and aromatic chemicals.

8. A process for degrading hazardous chemicals in situ or in a bioreactor, wherein said hazardous chemicals are selected from the group consisting of chloroaliphatic and aromatic chemicals, which comprises contacting said hazardous chemicals with an effective degrading amount of a recombinant microbe comprising a gene(s) obtainable from the microbe defined in claim 5, wherein said gene(s) encodes enzyme(s) having the property of degrading hazardous chemicals selected from the group consisting of chloroaliphatic and aromatic chemicals.

9. The process, according to claim 8, wherein said hazardous chemicals are selected from the group consisting of trichloroethylene, 1,1-dichloroethylene, cis-1,2 dichloroethylene, trans-1,2-dichloroethylene, vinyl chloride, toluene, phenol, o-cresol, m-cresol, o-xylene, and benzene.

10. The process, according to claim 8, wherein said hazardous chemicals are degraded in situ or in a bioreactor.

11. The process, according to claim 10, wherein said in situ degradation is selected from the group consisting of contaminated aquifers, landfills, industrial sites contaminated with hazardous chemicals, and hazardous chemicals waste collection areas.

12. A process for degrading hazardous chemicals in situ or in a bioreactor, wherein said hazardous chemicals are selected from the group consisting of chloroaliphatic and aromatic chemicals, which comprises contacting said hazardous chemicals with an effective degrading amount of an enzyme(s) produced by the microbe defined in claim 5, wherein said enzyme(s) has the property of degrading hazardous chemicals selected from the group consisting of chloroaliphatic and aromatic chemicals.

13. A process for isolating transposon induced tomA mutants of Pseudomonas cepacia G4 which comprises:

(a) transposon mutagenesis by a triparental mating comprising E. coli JM109 (pRZ102), E. coli JM109 (pRZ2013) and P. cepacia;

(b) selection for P. cepacia colonies containing a transposed copy of Tn5 by growth of the mating mixture on basal salts medium comprising sodium lactate and kanamycin sulfate;

(c) transferring selected P. cepacia colonies of (b) to nitrocellulose filters previously soaked in an aqueous solution of TFMP and air dried; and,

(d) isolating P. cepacia G4 5223 which lacks the ability to transform TFMP and to degrade TCE.

14. A process for isolating a constitutive P. cepacia G4 TCE degrader which comprises:

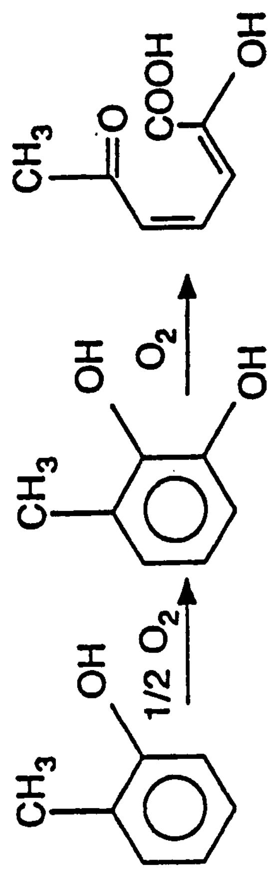
(a) inoculating transposon mutants of tomA by patching to basal salts plates comprising phenol as the sole carbon source;

(b) picking rare bacterial colonies to basal salts medium containing sodium lactate as the sole carbon source;

(c) pulling said rare bacterial colonies to nitrocellulose discs impregnated with TFMP; and,

(d) isolating bacterial colonies that turned yellow.

FIGURE 1



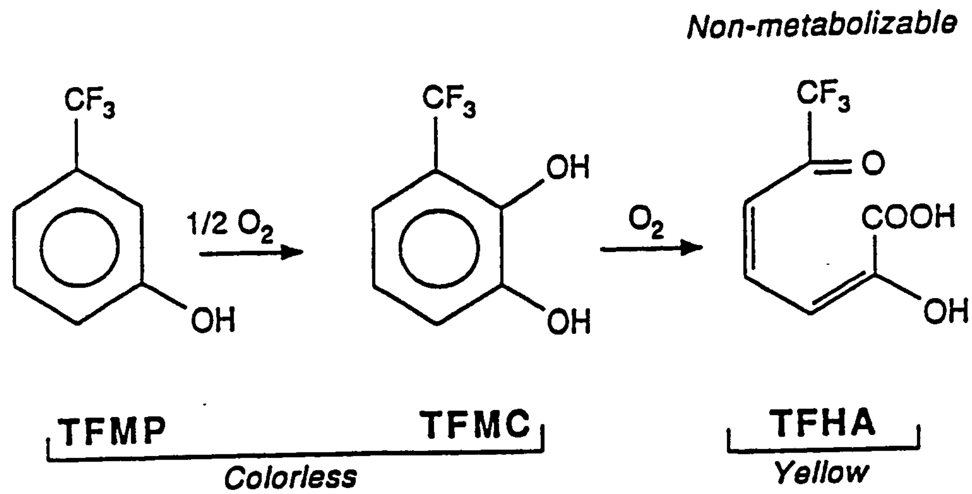

Non-metabolizable
CF3
OH
1/2 O2
O2
O
COOH
TFMP
TFMC
TFHA
Colorless
Yellow

FIGURE 2

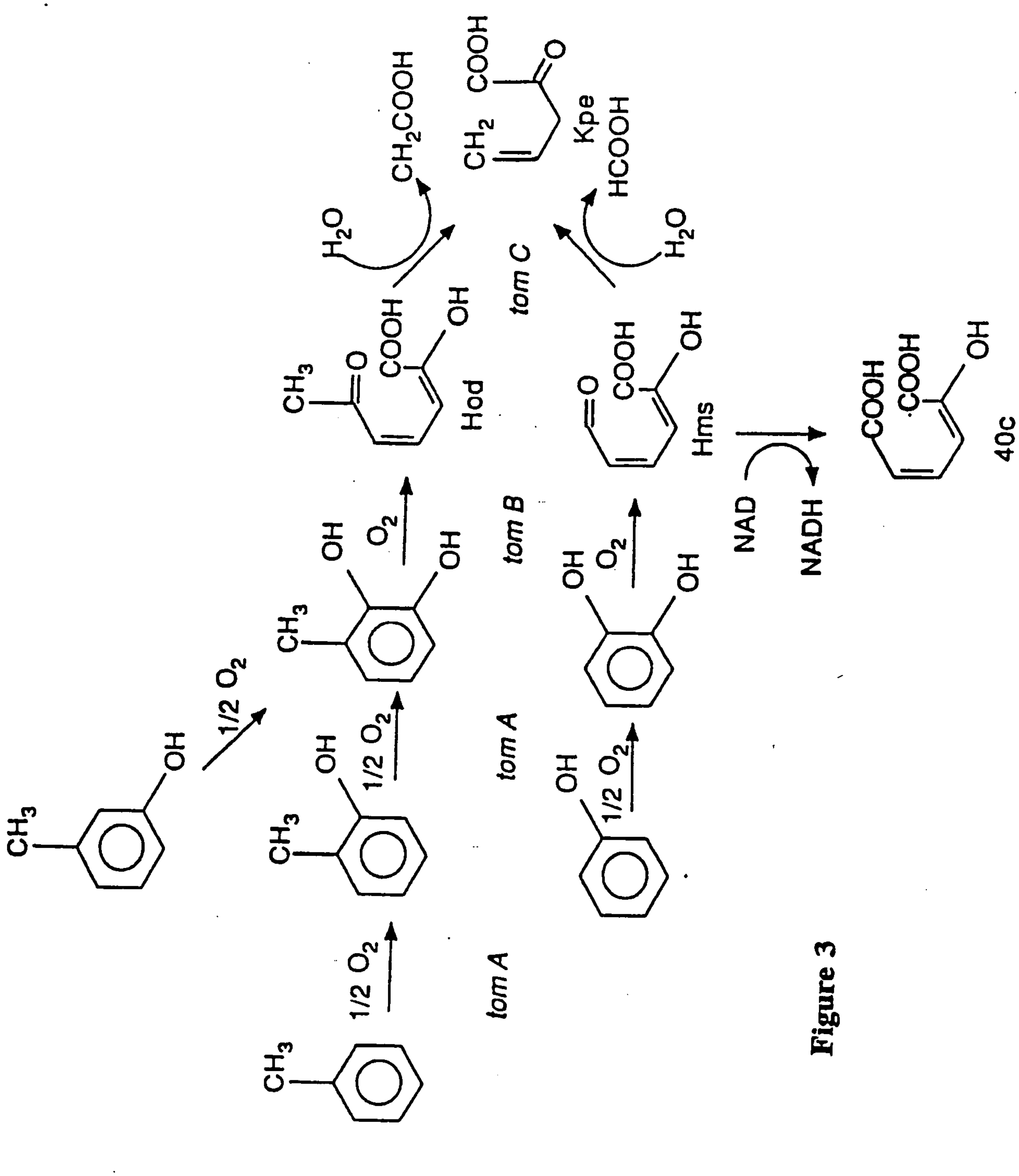

CH3
1/2 O2
tom A
OH
tom B
O2
COOH
Hod
Hms
H2O
CH2COOH
tom C
HCOOH
Kpe
NAD
NADH
40c

Figure 3

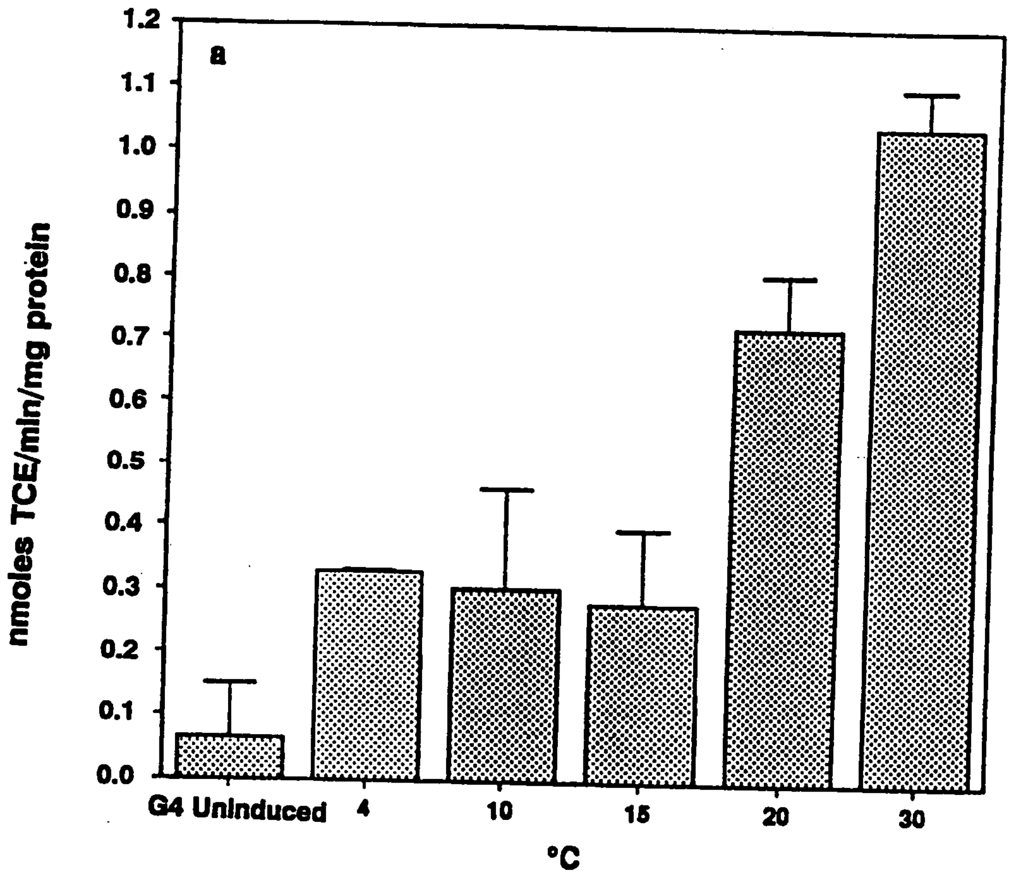

1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
nmoles TCE/min/mg protein
G4 Uninduced
4
10
15
20
30
°C

Figure 4a

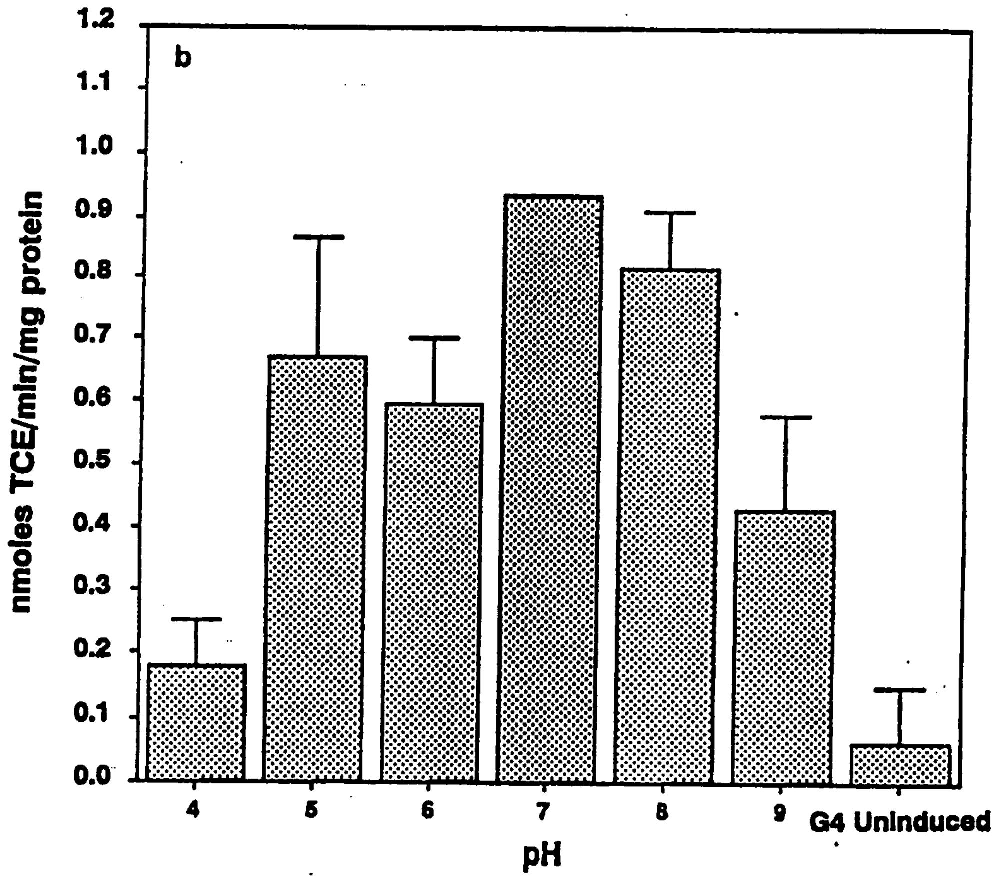

1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
b
nmoles TCE/min/mg protein
4
5
6
7
8
9
G4 Uninduced
pH

Figure 4b

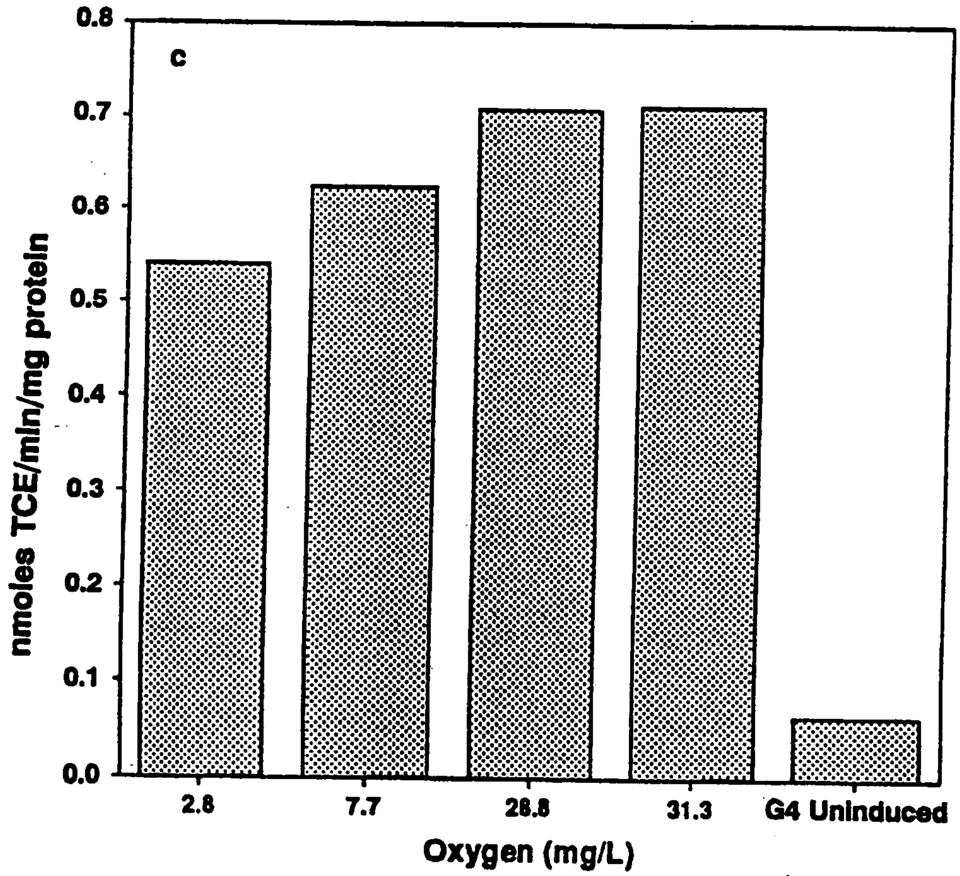
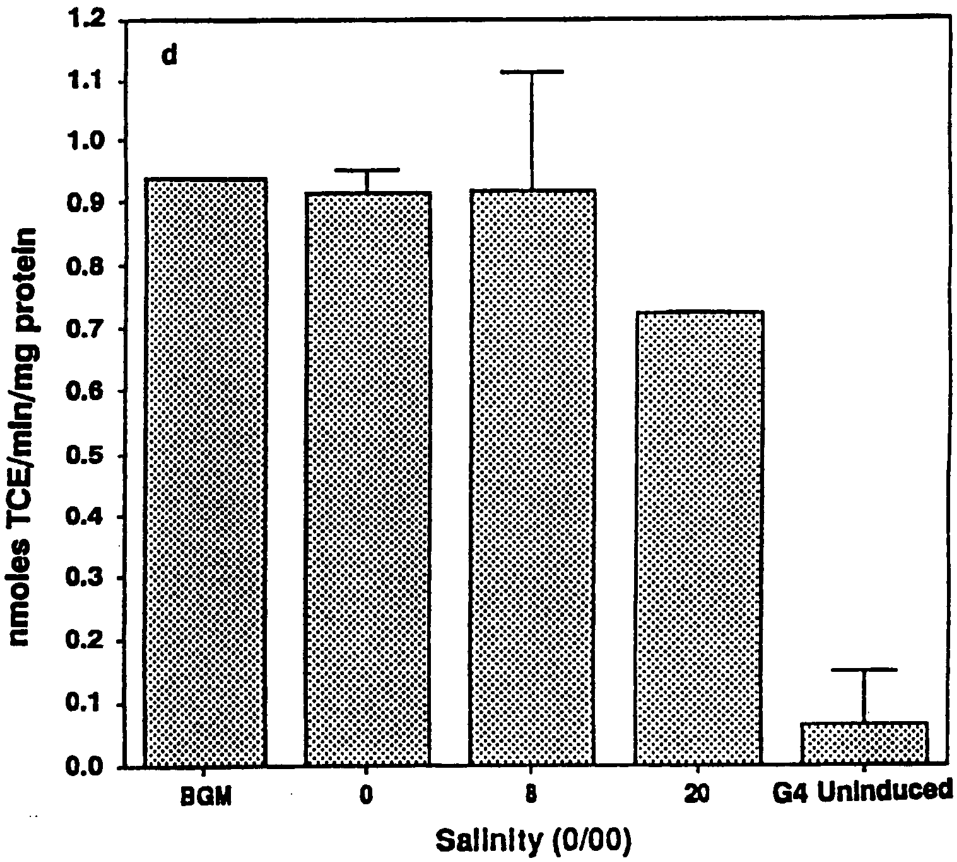

c
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
nmoles TCE/min/mg protein
2.8
7.7
28.8
31.3
G4 Uninduced
Oxygen (mg/L)

Figure 4c



d
nmoles TCE/min/mg protein
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
BGM
0
8
20
G4 Uninduced
Salinity (0/00)

Figure 4d

International Application No PCT/US 92/03515

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/31; C02F3/12; C12N9/02; C02F3/34
C12N15/52; C12N1/20; //(C12N1/20,C12R1:38)

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C02F ; C12N ; C12R

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	BIOTECHNOLOGY vol. 7, no. 3, March 1989, NEW YORK US pages 282 - 285; B. WINTER ET AL.: 'Efficient degradation of trichloroethylene by a recombinant Escherichia coli' see the whole document ---	7-10
X	APPLIED AND ENVIRONMENTAL MICROBIOLOGY vol. 53, no. 5, May 1987, WASHINGTON DC US pages 949 - 954; M. NELSON ET AL.: 'Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway' see page 951, column 2, paragraph 3 ---	6
A	US,A,4 925 802 (NELSON ET AL.) 15 May 1990 see the whole document ---	1-12
	-/--	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
31 AUGUST 1992	09. 09. 92
International Searching Authority	**Signature of Authorized Officer**
EUR PEAN PATENT OFFICE	CUPIDO M.

4

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	JOURNAL OF GENERAL MICROBIOLOGY v l. 131, no. 9, 1 September 1985, LONDON, GB pages 2449 - 2467; C. BOUCHER ET AL.: 'Transposon mutagenesis of Pseudomonas solanacearum: Isolation of Tn5-induced avirulent mutants' see the whole document ---	13,14
Y	ARCHIVES OF MIROBIOLOGY vol. 149, 1988, pages 188 - 197; K. ENGESSER ET AL.: 'Bacterial metabolism of side chain fluorinated aromatics' see the whole document ---	13,14

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9203515 SA 60209

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent ffice is in no way liable for these particulars which are merely given for the purpose of information. 28/08/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4925802	15-05-90	AU-B- 623211	07-05-92
		AU-A- 4810690	10-07-90
		EP-A- 0449957	09-10-91
		JP-T- 4502277	23-04-92
		WO-A- 9006901	28-06-90

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For more details about this annex : see Official Journal of the European Patent ffice, No. 12/82